

# Biomedical Technologies Inc.

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## RAT OSTEOCALCIN EIA KIT

Catalog No: **BT- 490**

96 Well Tests Storage 4°C

**\* See Storage Exception on Page 2**

For the Measurement of Rat Osteocalcin in Serum or Heparinized Plasma, cell culture and bone extracts.

### Introduction

Osteocalcin, the vitamin K-dependent protein of bone, is a specific product of the osteoblast. It is distinguished by its small size (5800 daltons) and the presence of gamma-carboxy-glutamic acid (Gla). In the presence of ionic calcium, the Gla residues allow a specific conformational change in the protein, which in turn promotes osteocalcin binding to bone mineral and subsequent accumulation in bone matrix. While osteocalcin is primarily deposited into the extracellular matrix of bone, a small amount can be detected in the blood. Circulating osteocalcin is thought to reflect that portion of newly synthesized protein that does not bind to bone but is released directly into the circulation.

### Principle of the Assay

This sandwich ELISA Kit is specific for rat osteocalcin only. Both carboxylated and decarboxylated rat osteocalcin are recognized. Rat osteocalcin can be measured directly from serum, heparinized plasma, bone extracts and serum free cell culture supernates. A monoclonal antibody directed against the N-terminal region of osteocalcin is bound to the polystyrene wells. After an incubation with sample, the plate is washed followed by an incubation with a second antibody (Goat polyclonal) of high specificity for the C-terminus of rat osteocalcin. Detection is achieved by a third incubation using a Horseradish Peroxidase conjugate of a Donkey anti-Goat IgG and subsequent enzyme assay. The osteocalcin concentration is proportional to color development. Standards of highly purified rat osteocalcin are used to generate a standard curve.

### References

1. Hauschka, P.V., Lian, J.B., Cole, D.E.C. and Gundberg, C.M.. Osteocalcin and Matrix Gla Protein: Vitamin K-Dependent Proteins in Bone. Physiological Reviews, 69: 990-1047 (1989).
2. Price, P.A. Williamson, M.K. and Lothringer, J.W. Origin of the Vitamin K-dependent Bone Protein Found in Plasma and Its Clearance by Kidney and Bone. Journal of Biological Chemistry, 256: 12760-12766 (1981).
3. Finkelman, R.D., Linkhart, T.A., Subburaman, M., Lau, K.H.W., Baylink, D.J. and Bell, N.H. Vitamin D Deficiency Causes a Selective Reduction in Deposition of TGF-beta in Rat Bone: Possible Mechanism for Impaired Osteoinduction. PNAS 88: 3657-3660 (1991).
4. Fante, P., Kindy, M.S., Mohapatra, S., Klein, J., Colombo, G., and Malluche, H.H. Dose-dependent effects of aluminum on osteocalcin synthesis in osteoblast-like ROS 17/2 cells in culture. Am. J. Physiol. 263 (Endocrinol. Metab. 26): E1113-E1118 (1992).
5. Zeni, S., Gomez-Acotto, C., Di Gregorio, S., and Mautalen, C. Differences in Bone Turnover and Skeletal Response to Thyroid Hormone Treatment Between Estrogen-Depleted and Repleted Rats. Calcif Tissue Int. 67: 173-177 (2000).
6. Maccarinelli G., Sibilila V., Torsello A., Raimondo F., Pitto M., Giustina A., Netti C. and D. Cocchi. Ghrelin regulates proliferation and differentiation of osteoblastic cells. J. of Endocrinology, 184 : 249-256 (2005).

**FOR RESEARCH USE ONLY (REV. 11/10)**

## Reagents: Description and Preparation

Store all reagents at 4°C up to 6 months except as noted.

*\* See Storage Exception*

**CAUTION:** DO NOT USE AZIDE.

1. Working sample buffer **BT- 471R**. One 60ml bottle and one 125ml bottle.
2. Phosphate-Saline Concentrate (wash buffer) **BT- 492**. One 100ml bottle. Transfer contents to a graduated cylinder, and bring volume up to 500ml with deionized water.
3. Osteocalcin Standard **BT- 493**. One vial 200ng lyophilized. Reconstitute vial with exactly 2.0ml of sample buffer. Use for making working standards. Store at -20°C.
4. Osteocalcin Antiserum Concentrate. **BT- 494**. One vial containing 0.5ml. Dilute contents to 10ml with sample buffer and use immediately. This is a 1:20 dilution. Dilute only enough antibody for current use.
5. Donkey anti-Goat IgG Peroxidase **BT- 495**. 2 vials, lyophilized. Reconstitute 1 vial with 12ml of sample buffer 30 minutes before use. Discard all leftovers. Store the second vial at 4°C for later use if needed.
6. Peroxidase Substrate TMB (3,3<sup>1</sup>,5,5<sup>1</sup>-tetramethyl benzidine) **BT- 497**. One 6ml vial.
7. Hydrogen Peroxide Solution **BT-498**. One 6ml vial.
8. Stop Solution **BT- 499**. One 12ml vial.
9. Rat Serum Control **BT-496**. 50ul lyophilized whole serum. Reconstitute with 50ul deionized water, gently mixing 15-20 minutes. Dilute with 0.45ml sample buffer, mix thoroughly. Multiply the quantity printed on the vial by 2 to obtain the ng/ml. Store at -20°C.
10. One 96 well plate (8 well removable strips) coated with osteocalcin antibody. Extra sealing tape provided.

### **Other Supplies Required**

1. ELISA Plate Reader which can measure absorbance at 450nm.
2. Pipettes: micropipettes 5-1000ul.
3. A plate washer is recommended for washing.
4. A 37°C Incubator.
5. Deionized water.

### **Precautions**

Some components of this kit contain isothiazolones (5ppm) as preservative. Stop solution contains hydrochloric and phosphoric acids. Keep all materials away from the skin and eyes.

## Sample Preparation

Aliquot and freeze immediately all samples for future analysis. Samples stored frozen are stable for six months. Avoid repeated freeze-thawing of samples, osteocalcin levels will decline considerably. Samples from serum, heparinized plasma and cell culture media can be measured directly using BTI Rat Osteocalcin ELISA Kit. Samples containing azide cannot be assayed.

### Rat Serum

Rat serum must be diluted at least 10 fold with sample buffer. We recommend a 1/10 to 1/20 dilution with sample buffer. Thus it is possible to quantitate osteocalcin in 5ul of rat serum (10ul in duplicate). Serum samples can conveniently be aliquoted in 25-50ul amounts and stored at -70°C.

The design of the animal experiment is most important. Many variables effect serum osteocalcin levels: age, growth rate, hormonal status, vitamin D intake, stress, circadian rhythm, etc. It is desirable to take blood samples under the same conditions: time of day, without stress, etc. and process the serum (or plasma) the same way for each sampling.

### Rat Serum Osteocalcin Levels

The following results were obtained with male Spraque Dawley rats of given ages. (Blood was allowed to clot at room temperature for 15 minutes and then 15 minutes at 4°C. Serum was drawn off and frozen immediately.)

<u>Age</u>	<u>ng/ml Serum Osteocalcin</u>
1 month	78
3 months	45
5 months	17

A serum sample went through 2 freeze-thaw cycles and was incubated at room temperature for 4 hours without any loss of osteocalcin concentration.

### Rat Bone

Typical levels are 1.5-2.0ng osteocalcin/ug dry bone powder. EDTA extracts of bone powder can be assayed after appropriate dilution.

### Rat Osteoblast Culture Medium

Concentrations of osteocalcin in conditioned media range from <1ng/ml to 400ng/ml depending on the cell type and culture conditions. It is best to wash cells and grow in serum free media (24-48 hours) prior to measuring osteocalcin levels. Bovine serum products (Bovine IgG and osteocalcin) can interfere in the assay. Many researchers find that osteocalcin synthesis can be stimulated in primary bone cell cultures with  $10^{-9}$ M 1,25 dihydroxy Vitamin D<sub>3</sub>. Full dilution series should be done to establish linearity of dilution. Medium and serum interference should be assessed by appropriate blanks and internal standards of medium with added rat osteocalcin.

# Assay Procedure

**CAUTION: KEEP AZIDES AWAY FROM ALL SOLUTIONS AND SAMPLES**

**All Reagents must be at room temperature prior to use.**

## PROCEDURE

1. Please refer to page 2 for preparation of reagents and page 3 for sample preparation.
2. Remove microtiter plate from resealable bag. Strips not used should be removed from the frame and resealed in the bag for future use.
3. Dilute the stock standard (100ng/ml) in polypropylene tubes with sample buffer to give six or seven standards in the range of 0.25 to 20ng/ml. 0.33, 1.0, 2.5, 5.0, 10 and 20ng/ml results in a good curve.
4. Pipet 25ul of sample buffer (Blank), Standards, Controls and Unknowns into designated duplicate wells followed by 100ul of osteocalcin antiserum in each well. Cover tightly with plastic seal provided, incubate at **37°C, 2.5 hours**.
5. Aspirate wells completely and wash the plate 3 times (plate washer) or 5 times (by hand) with 0.3ml/well Phosphate-Saline wash buffer. (Complete removal of wash buffer after each wash is important for good reproducibility).
6. Add 100ul of the diluted Donkey anti-Goat IgG Peroxidase to each well. Incubate at **room temperature for 1 hour**.
7. Mix one volume of TMB solution (**BT- 497**) with one volume of Hydrogen Peroxide solution (**BT- 498**) and put aside. (Only mix an amount sufficient for the number of wells in use) Wash the plate as in step 5. Immediately add 100ul of substrate mix to all wells and incubate at **room temperature, in the dark for 30 minutes**.
8. Add 100ul of Stop Solution to all wells, swirl and measure absorbance at 450nm within **15 minutes**.

## **Calculation of Results**

Average duplicates for all determinations. Subtract the (Blank) from all average readings. Plot net optical density of the standards vs. log of the concentration of each, draw the best curve. Obtain concentration of each unknown from this standard curve. Always generate a standard curve for each new assay.

## **Specifications**

Sample size: 25ul  
Assay time: 5 hrs  
Sensitivity: 0.5ng/ml  
Working range: 0.33-20ng/ml  
Intraassay variation: 4% (95% limits)  
Interassay variation: 7% (95% limits)

## **Typical Data (Do Not Use for determination of Unknowns)**

<b>ID</b>	<b>A<sub>450</sub></b>	<b>Average – Blank</b>
Standards:		
(Blank) 0ng/ml	.201	
(Blank)	.196	(.199)
.33ng/ml	.308	
.33ng/ml	.272	.091
1ng/ml	.510	
1ng/ml	.486	.300
2.5ng/ml	.771	
2.5ng/ml	.856	.615
5ng/ml	1.120	
5ng/ml	1.157	.940
10ng/ml	1.384	
10ng/ml	1.375	1.181
20ng/ml	1.539	
20ng/ml	1.604	1.373

**Typical Standard Curve**

